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SCREENING OF IMMUNOENHANCING DRUGS WITH ANTIVIRAL ACTIVITY AGAINST MEMBERS OF THE ARENA-, ALPHA-, AND ADENOVIRIDAE

Annual Report

February 13, 1989

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Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6121

University of Alabama
Tuscaloosa, Alabama 35487-1969

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION The University of Alabama 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Tuscaloosa, Alabama 35487-1969	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-86-C-6121
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 63763A; PROJECT NO. 3M2-63763D807; TASK NO. AE; WORK UNIT ACCESSION NO. 377

11. TITLE (Include Security Classification)
(U) Screening of Immunoenhancing Drugs with Antiviral Activity Against Members of the Arena-, Alpha-, and Adenoviridae

12. PERSONAL AUTHOR(S)
Paul A. LeBlanc and Alvin L. Winters

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 1/15/88 TO 1/14/89	1989 February 13	27

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	RA 1,
06	04		
06	03		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostian	22b. TELEPHONE (Include Area Code) 301-663-7325 22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985.

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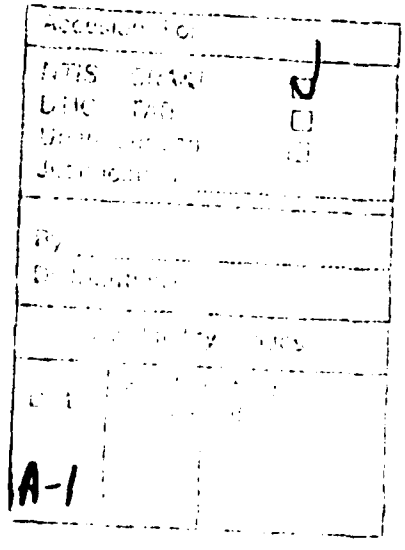

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INTRODUCTION

This project was designed to test a series of immunomodulatory compounds designated by the U.S. Army Research and Development Command for their ability to protect animals from an otherwise lethal infection of virus and to determine the mode of action of useful compounds. During this third year of the contract emphasis was placed on two areas. These were the development of virally infected targets for use in in vitro assays, determine if these targets were handled in the same manner as other targets traditionally used for in vitro testing of macrophages and cytotoxic T lymphocyte (CTL) responses, and measurement of the effect of the compounds on the CTL response.

Materials and Methods

Endotoxin Detection.

All reagents were tested for the presence of bacterial endotoxin by the gel formation. Lymulus amebocyte lysate (LAL) test (1). The LAL was obtained from Associates of Cape Cod (Woods Hole, MA) with a 0.03 endotoxin unit per ml sensitivity. The sensitivity was confirmed with each test by the titration of standard lipopolysaccharide (LPS). Materials that test negative in this test were defined as endotoxin free.

Cells and Cell Culture.

Modified Eagles' minimal essential medium (HMEM) Ham's F12 and Dulbeco's modified Eagles' medium (DMEM) were prepared from a powdered mix (HyClone Laboratories, Logan, UT) and supplemented with glutamine (2 mM, Flow Laboratories, McClean VA), sodium bicarbonate (2 mg/ml, Sigma Chemical Co., St. Louis, MO), and HEPES (15 mM, Research Organics, Cleveland, OH). All serum, including fetal bovine serum (FBS) was obtained from Hyclone Laboratories. Clone 929 of L-cells (CCL 1) were obtained from the ATCC, Rockville, Maryland, and were propagated in HMEM + 10% FBS. Vero (African green monkey kidney) BALB/c3T3 cells were obtained from Dr. Gillespie (University of North Carolina Cancer Center, Chapel Hill, NC) and propagated in HMEM +2% FBS or a 1:1 mixture of F12 and DMEM with 10% FBS, respectively. Chicken embryo cell (CEC) were produced by trypsinization of 9-11 day old chicken embryos according to the method of Scherer (2) except that HMEM + 10% FBS or HMEM + 4% chicken serum were used as growth media. The preparation of bone marrow culture derived macrophages has been previously been described (3).

Viruses.

Venezuelan equine encephalitis (VEE) strain 68U201 (4) was obtained from Dr. Peter Jahrling, USAMRIID, and was propagated in primary chicken embryo cell culture to prepare a working stock. Pichinde strain An3739 (5) was obtained from the same source and was propagated in Vero cell cultures to prepare a working stock of virus. Pichinde strain An4763 (6) was obtained from the Southern Research Institute and propogated in Vero cells. Vesicular stomatitis virus (VSV) was propogated in L-929 cells.

Mixed cell bed cultures were prepared by the addition of 5 ml of HMEM supplemented with 5% heat inactivated calf serum containing 9 x 10^5 Vero and 9 x 10^5 L-929 cells to a 60 mm tissue culture dish. The dish was incubated overnight and then overlaying medium removed and the cells infected by the addition of 0.5 ml of an appropriate 10-fold dilution of

Pichinde virus or plaque progeny. After 1 hour for adsorption, the cultures were overlayed with 8 ml of HMEM supplemented with 5% heat inactivated calf serum and 1% agarose. A neutral red containing overlay medium was added on day 4 and plaques were observed and or picked on days 5 or 7. Plaques progeny were isolated by the removal of an agarose plug from the overlaying medium directly over the center of the desired plaque using a sterile pasteur pipet cut to give an outside diameter of 2.5 mm. The agar plug was emulsified in 1.0 ml of medium. The resultant plaque progeny were then further purified by plaquing 10-fold serial dilutions on duplicate cultures of the appropriate cell type and reselecting plaques from dishes developing only a few, well-isolated plaques.

In Vivo Tests.

A compound was administered as recommended by the source (dosages, times, and routes are indicated in text) and then the mice were challenged with 400 pfu of the 68U201 strain of VEE, subcutaneously. Mice were observed daily for deaths for 31 days.

Cytotoxicity Assays.

Compounds were tested for their ability to modify the cytotoxic T cell response generated due to the injection of either the TC-83 strain of Venezuelan equine encephalitis (VEE) virus or to the histoincompatible cell line P815. Groups of C3H/HeN ($H\text{-}2^k$) mice were treated with a compound or with a placebo and then challenged with either subcutaneously with 10^4 pfu of VEE strain TC-83 grown in chicken serum or intraperitoneally with 10^6 P815 ($H\text{-}2^d$) cells, or not injected with an antigen. Eleven days later, mice were sacrificed and a single cell suspension prepared from their spleens. The spleen cells from all mice in a group were pooled, and the pool tested at various effector to target ratios against four different ^{51}-Chromium labelled target cells. The target cells were VEE-infected L929 cells (histocompatible, virus specific), Vesicular stomatitis virus (VSV) infected L929 cells (histocompatible, virus nonspecific), P815 cells (allogeneic challenge), and VEE infected BALB/c3T3 cells (histoincompatible, virus specific). BALB/c3T3 (3T3) cells are of the same H-2 type as P815 and these two cells served as mutual internal controls. Further, changes in natural killer cell (NK) activity could be addressed by comparing the results obtained from spleen cells obtained from control and treated animals and tested with L929-VSV cells or 3T3-VEE cells. P815 cells are relatively NK resistant. The data from all ^{51}Cr-release assays were expressed as specific release based on the formula: percent specific release =
(experimental - spontaneous) / (maximum - spontaneous) x 100.

Macrophage activation assays were performed as previously described (7) except that virus-infected BALB/c3T3 cells were used as targets. These cells were used because the cells are histoincombatible with the C3H/HeN macrophages used. Further, uninfected BALB/c3T3 cells are seen as normal by macrophages and not lysed, while either transformed or infected cells make satisfactory targets. Bone marrow culture-derived macrophage monolayers, prepared in 96-well plates were treated with 0.1 ml of various stimuli, as described in the text, for either 4 or 24 hrs. Each stimuli was tested in triplicate. The stimuli were then removed and the monolayers washed twice with warm H-MEM + 10% FBS. Targets were added to yield 2 x 10^4 ^{51}Cr-labelled targets in a total volume of 0.2 ml in each well. The plates were incubated at 37° in a moist 5% CO_2 in air atmosphere for sixteen hr.

The uppermost 0.1 ml of medium was removed from each well and the amount of released radioactivity determined in an automatic gamma spectrometer.

Targets used were: P815, BALB/c3T3, Vero, an African green monkey kidney cell, vesicular stomatitis virus (VSV) infected BALB/c3T3 (3T3-VSV), VSV-infected Vero (Vero-3T3), and Venezuelan equine encephalitis virus, strain TC-83, (VEE) -infected BALB/c3T3 (3T3-VEE). P815 cells were suspended into the overlaying medium and counted. Uninfected BALB/c and Vero cells were prepared by trypsinization of near confluent monolayers and washed by centrifugation in H-MEM + 10% FBS. Near confluent monolayers of either BALB/c3T3 or Vero cells were infected with a multiplicity of infection (MOI) of 3. After 45 min at 37° for virus adsorption, monolayers were washed once with H-MEM + 10% FBS and once with H-MEM to remove unadsorbed virus. The cells were then removed from the flask by trypsinization and washed by centrifugation with H-MEM + 10% FBS. All cells were labelled by exposing 1.2×10^7 cells to 0.275 mCi of ^{51}Cr as sodium chromate (Amersham, Arlington Heights,IL) in a total volume of 1.0 ml for 90 minutes. Cells were assessed for viability by trypan blue exclusion just before addition to the assay plate and were >95% viable.

Bacterial lipopolysaccharide (LPS) was phenol extracted and purified (lipid A rich, fraction II) [19] from Escherichia coli 0111:B4 and was the generous gift of Dr. D. C. Morrison (Kansas University Medical Center, Kansas City, KS). Gamma interferon (γIFN) was recombinant rat gamma interferon (Amgen Biological, Thousand Oaks, CA). Alpha (α-IFN) and beta (β-IFN) interferons were purchased from Lee Biomolecular, San Diego, CA (#20061, Lot 83002, 2.7×10^6 IRU/mg; #20181, Lot 83055, 1.8×10^8 IRU/mg, respectively). A mixture containing both α-IFN and β-IFN (α/β-IFN) was prepared by mixing equal activities of each purified interferon. This mixture was used for most experiments because preliminary experiments showed little difference in activity between α-IFN and β-IFN, which was in agreement with previous reports (8). Interferon (IFN) preparations were assayed for antiviral activity by plaque reduction of VSV on monolayers of L-929 cells (8,9). Each dilution was tested in triplicate and activity was expressed in International Reference Units (IRU), with mouse fibroblast interferon as a reference standard (NIH). Anti-mouse IFN (Alpha and Beta, 1×10^4 IRU/ml) globulin in phosphate buffered saline was lot # 84005 of catalog # 21031 from Lee Biomolecular.

Inhibition studies were conducted in a similar manner except that targets were added in medium containing the inhibitors.

Tumor Necrosis Factor mRNA Production

The production of Tumor necrosis factor alpha (TNF) mRNA by bone marrow culture derived macrophages was determined by probing with a plasmid containing a 1700 bp fragment of TNF cloned into the polylinker of PUC 9 and obtained from Dr. Steven Taffet, SUNY Upstate Medical Center, Syracuse, NY. The probe was labelled with $^{32}PdCTP$ (Amersham, Arlington Heights, IL) by nick translation using a commercial kit (BRL, Gaithersburg, MD) and detected by radioautography.

Macrophages (1×10^7) were exposed to various stimulatory conditions, as described in the text, and then chilled, scrapped from the dish and pelleted. The cells were lysed with NP-40 and the nuclei pelleted. The RNA was then extracted from the supernatant and blotted onto nitrocellulose filter for probing by previously described methods (10). The intensities of the dots were determined by optical density scanning of the dried films.

Target Sensitivity to TNF

Recombinant murine TNF-α (rTNF) was obtained from Genzyme (Boston, MA). Chromium-labelled target cells (1×10^4) were exposed to increasing concentrations of rTNF in a total volume of 200 μl HMEM + 10% FBS for 16h. Kill was measured by determining the amount of specific chromium release usinf the formula given under cytotoxic assays. The ability of anti-TNF antibody (Genzyme) to block cytotxicity was determined using a standard ^{51}Cr-release assay described above, except that the targets were added in the presence of the anti-TNF antibody.

RESULTS AND DISCUSSION

Macrophage cytotoxicity for virally infected cells

Bone marrow culture derived macrophages demonstrated little cytotoxicity for uninfected BALB/c3T3 (Table 1) even after stimulation with LPS and/or IFN. On the other hand, viral infection of these cells by VSV rendered them susceptible to lysis by macrophages. This cytolytic activity could be augmented by the pretreatment of the macrophages with LPS, γ-IFN, α/β-IFN, or either IFN plus LPS. This pattern was repeated using another cell line, Vero, as a target (Table 2). Further, the ability of macrophage monolayers to mediate cytotoxicity was not restricted to cells infected with VSV. Similar results were obtained from BALB/c3T3 infected by VEE instead of VSV (Table 3).

These initial studies appeared to show a different level of responsiveness to activating signals for cytotoxicity for virally infected cells than has been seen for tumor targets. Therefore, monolayers were treated in parallel and allowed to react with either a virally infected or

TABLE 1. Activation of Macrophages for Cytolysis of VSV-Infected BALB/c3T3 Target Cells

Treatment[a]	Percent Specific ^{51}Cr-Release[b]	
	BALB/c3T3	3T3-VSV[c]
Medium	-6	16
LPS (ng/ml)		
10	-4	110
1	-4	35
0.1	-5	13
0.01	-4	2
0.001	-5	11
γ-IFN (10 IRU/ml)	-5	46
γ-IFN + LPS (10 IRU/ml + 1 ng/ml)	-7	65
α/β-IFN (500 IRU/ml)	-7	61
α/β-IFN + LPS (500 IRU/ml + 1 ng/ml)	-6	45

[a]Macrophages were exposed to activators for 4 hr, washed with warmed medium, and then coincubated with ^{51}Cr-labelled target cells for 16 hr.
[b]Mean of triplicate determinations.
[c]Cells were infected with VSV at an MOI of 3 before labelling with ^{51}CR.

TABLE 2. Activation of Macrophages for Cytolysis of VSV-Infected Vero Target Cells

Treatment[a]	Percent Specific ^{51}Cr-Release[b]	
	Vero	VSV-Vero[c]
Experiment 1		
Medium	1	12
LPS (3 ng/ml)	0	29
α/β-IFN (100 IRU/ml)	0	30
τ-IFN (100 IRU/ml)	0	26
Experiment 2		
Medium		15
LPS (10 ng/ml)		40
1		35
0.1		26
0.01		12
τ-IFN (1 IRU/ml)		10
τ-IFN + LPS (1 IRU/ml + 1 ng/ml)		23
α/β-IFN (500 IRU/ml)		26
α/β-IFN + LPS (500 IRU/ml + 1 ng/ml)		33

[a]Macrophages were exposed to activators for 4 hr, washed with warmed medium, and then coincubated with ^{51}Cr-labelled target cells for 16 hr.
[b]Mean of triplicate determinations.
[c]Cells were infected with VSV at an MOI of 3 before labelling with ^{51}Cr.

TABLE 3. Activation of Macrophages for Cytolysis of VEE-Infected BALB/c3T3 Target Cells[a]

Treatment[b]	Percent Specific ^{51}Cr-Release[c]
Medium	2
LPS (10 ng/ml)	16
LPS (0.01 ng/ml)	6
τ-IFN (1 IRU/ml)	7
τ-IFN + LPS (1 IRU/ml + 10 ng/ml)	22
τ-IFN + LPS (1 IRU/ml + 0.01 ng/ml)	13
α/β-IFN (500 IRU/ml)	9
α/β-IFN + LPS (500 IRU/ml + 10 ng/ml)	14
α/β-IFN + LPS (500 IRU/ml + 0.01 ng/ml)	9

[a]Cells were infected with VEE at an MOI of 3 before labelling with ^{51}Cr.
[b]Macrophages were exposed to activators for 4 hr, washed with warmed medium, and then coincubated with ^{51}Cr-labelled target cells for 16 hr.
[c]Mean of triplicate determinations.

tumor targets. Figure 1 shows the results of two independent experiments comparing the cytolytic activity of bone marrow culture-derived macrophages for either VSV-infected BALB/c3T3 cells or P815 mastocytoma cells. The P815 cell line was chosen as a representative tumor cell target [20-22]. When comparing the ability of various stimulators to augment macrophage cytotoxicity against each target cell, it is apparent that lower doses of

LPS were able to stimulate activity against virally infected targets better than activity against tumor targets. This is particularly noticeable at

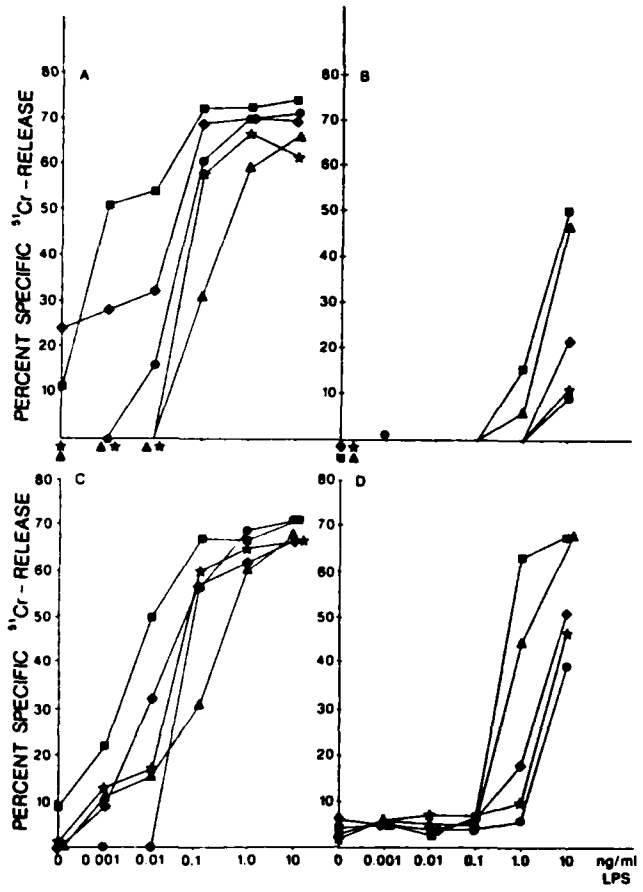


FIGURE 1. Comparison of activation of bone marrow culture-derived macrophages for cytolysis of virally infected or tumor targets. Macrophage monolayers were exposed for four hours to varying doses of LPS alone (●), or incombination with 500 IRU/ml α/β-IFN (■), 50 IRU/ml α/β-IFN (◆), 10 IRU/ml γ-IFN (▲), or 1 IRU/ml γ-IFN (✱). The monolayers were then washed and cocultivated with either 3T3-VSV (A and C) or P815 (B and D) targets. Panels A and B represent one experiment while C and D represent a second independent experiment. Each point represents the mean of triplicate determinations.

low (0.001-0.01 ng/ml) LPS concentrations and in the presence of α/β-IFN. While the addition of γ-IFN at 1 IRU/ml appeared to inhibit slightly the activity of LPS in activating macrophage monolayers against virally infected targets, this was a frequent but not constant finding.

Short vs. long preincubation with stimuli

The effect of the duration of exposure to stimuli on the ability to activate macrophage monolayers for killing virally-infected or tumor cells was investigated. Monolayers of bone marrow culture derived macrophages were exposed to activating stimuli for either 4 or 24 hr. The monolayers were then washed twice to remove stimuli and targets added. The results are shown in Tables 4-6. In general, the activity against the tumor targets was stable to variations in the time of exposure to stimuli. As expected, cytolytic activity for tumor targets was seen only with macrophage monolayers exposed to both interferon and LPS, for both long and short exposure to stimuli. For all stimulation condition, activity against virally infected target cells was substantially less when the monolayers were exposed to stimuli for 24 hr than when exposed to the same stimuli for only 4 hr. In fact, the level of cytolytic activity was often reduced to zero. The conditions showing the least decay in activity with 24 hr stimulation were monolayers treated with IFN alone. This was true for both VSV- and VEE-infected targets.

TABLE 4. Effect of Duration of Exposure to Stimuli on Bone Marrow Culture-Derived Macrophage Cytolysis of 3T3-VSV[a].

Treatment[b]	Percent Specific ^{51}Cr-Release[c] After Exposure to Stimuli for:	
	4 hr	24 hr
LPS, 10 ng/ml	62	0
LPS, 0.01 ng/ml	20	1
γ-IFN, 1 IRU/ml	39	12
γ-IFN + LPS, 1 IRU + 10 ng/ml	54	0
γ-IFN + LPS, 1 IRU + 0.01 ng/ml	26	10
α/β-IFN, 500 IRU/ml	44	15
α/β-IFN + LPS, 500 IRU + 10 ng/ml	59	10
α/β-IFN + LPS, 500 IRU + 0.01 ng/ml	74	11

[a]Cells were infected with VSV at an MOI of 3 before labelling with ^{51}Cr.
[b]Macrophages were exposed to activators for 4 or 24 hr, washed with warmed medium, and then coincubated with ^{51}Cr-labelled target cells for 16 hr.
[c]Mean of triplicate determinations.

TABLE 5. Effect of Duration of Exposure to Stimuli on Bone Marrow Culture-Derived Macrophage Cytolysis of P815.

Treatment[a]	Percent Specific ^{51}Cr-Release[b] After Exposure to Stimuli for:	
	4 hr	24 hr
LPS, 10 ng/ml	6	0
LPS, 0.01 ng/ml	0	0
γ-IFN, 1 IRU/ml	0	0
γ-IFN + LPS, 1 IRU + 10 ng/ml	74	70
γ-IFN + LPS, 1 IRU + 0.01 ng/ml	2	2
γ-IFN, 5 IRU/ml	0	0
γ-IFN + LPS, 5 IRU + 10 ng/ml	100	100
γ-IFN + LPS, 5 IRU + 0.01 ng/ml	6	11
α/β-IFN, 500 IRU/ml	6	0
α/β-IFN + LPS, 500 IRU + 10 ng/ml	21	44
α/β-IFN + LPS, 500 IRU + 0.01 ng/ml	0	0
α/β-IFN, 1000 IRU/ml	0	0
α/β-IFN + LPS, 1000 IRU + 10 ng/ml	36	57
α/β-IFN + LPS, 1000 IRU + 0.01 ng/ml	0	0

[a]Macrophages were exposed to activators for 4 or 24 hr, washed with warmed medium, and then coincubated with ^{51}Cr-labelled target cells for 16 hr.
[b]Mean of triplicate determinations.

TABLE 6. Effect of Duration of Exposure to Stimuli on Bone Marrow Culture-Derived Macrophage Cytolysis of 3T3-VEE[a]

Treatment[b]	Percent Specific ^{51}Cr-Release[c] After Exposure to Stimuli for: 4 hr	24 hr
LPS, 10 ng/ml	47	0
LPS, 0.01 ng/ml	5	4
γ-IFN, 1 IRU/ml	14	11
γ-IFN + LPS, 1 IRU + 10 ng/ml	20	0
γ-IFN + LPS, 1 IRU + 0.01 ng/ml	31	0
γ-IFN, 5 IRU/ml	23	11
γ-IFN + LPS, 5 IRU + 10 ng/ml	28	0
γ-IFN + LPS, 5 IRU + 0.01 ng/ml	34	0
α/β-IFN, 500 IRU/ml	21	0
α/β-IFN + LPS, 500 IRU + 10 ng/ml	19	0
α/β-IFN + LPS, 500 IRU + 0.01 ng/ml	22	0
α/β-IFN, 1000 IRU/ml	23	0
α/β-IFN + LPS, 1000 IRU + 10 ng/ml	16	0
α/β-IFN + LPS, 1000 IRU + 0.01 ng/ml	13	0

[a]Cells were infected with VEE at an MOI of 3 before labelling with ^{51}Cr.
[b]Macrophages were exposed to activators for 4 or 24 hr, washed with warmed medium, and then coincubated with ^{51}Cr-labelled target cells for 16 hr.
[c]Mean of triplicate determinations.

Effect of anti-IFN globulin

From the above data, one can conclude that there was a difference in the interaction between the macrophage monolayers and the different targets. Part of this difference appeared to be a greater sensitivity of macrophage monolayers to activating stimuli when measured with virally infected target cells as opposed to tumor target cells. One difference between the two types of target cells was that the virally infected target cells were producing interferon. This interferon may then have interacted with the macrophages and caused the difference in activity previously observed. In order to see if interferon produced by target cells might have been involved with the observed differences, macrophage monolayers were exposed to medium alone or medium containing 0.1 ng/ml LPS. This concentration of LPS was chosen because it gave consistently high levels of activity against VSV-BALB/c3T3 cells with little or no activity against P815 tumor targets. After exposure to the activating stimulus, the monolayers were washed and target were added in the presence of varying concentrations of antibody to α/β-IFN. The presence of interferon neutralizing antibody did not effect the ability of the LPS to activate the macrophage monolayers for anti-infected cell activity (Table 7). The addition of 2500 units/ml anti-IFN globulin actually resulted in the increase in activity of unstimulated monolayers. This activity was likely due to the level of contaminating endotoxin. Supernatants collected from parallel wells containing LPS-treated macrophages and virally infected but unlabeled target cells were harvested after 16 hr of incubation, acidified to pH 2 for 24 h, neutralized, and assayed for IFN activity. These supernatants contained approximately 1100 IRU IFN/ml. A 1:1 mixture of culture supernatant with anti-IFN serum was assayed for residual

neutralizing activity against 10 IRU α/β IFN. The mixture yield 50% neutralization to a dilution of 1:512.

TABLE 7. Effect of Anti-Interferon Serum on Macrophage anti-3T3-VSV Cytotoxicity[a]

Treatment[b]	Percent Specific ^{51}Cr-Release[c]
0.1 ng/ml LPS + anti-IFN (U/ml)	
0	91
2500	97
833	95
278	117
No activation + anti-IFN (U/ml)	
0	0
2500	36
833	-13
278	6

[a]Cells were infected with VSV at an MOI of 3 before labelling with ^{51}Cr.
[b]Macrophages were exposed to either medium or medium containing 0.1 ng/ml LPS for 4 hr, washed, and coincubated with target cells in the presence of the indicated concentration of anti-IFN antibody.
[c]Mean of triplicate determinations.

These findings demonstrate that while the activation of macrophages for killing virally infected cells shares many characteristics of activation for tumor cell killing, there are significant differences in sensitivity to activating stimuli. There are also qualitative differences in the persistence of the responses to long and short exposures to activating signals. Thus there appears to be the need to use virally infected cells rather than tumor cells as targets for measuring the effect of immunomodulators on macrophage activity for cytolysis since there are differences in the way these two targets are handled.

Requirements for macrophage cytolysis of tumor or virally infected targets.

We next looked at the relative contribution of various cytotoxic mechanisms known to be used by macrophages. The goal was to determine if virally infected cells and tumor cells are killed by the same mechanisms. From this information it would be possible to select appropriate parameters on which to concentrate as sites of effective antiviral immunomodulation. Three of the mechanisms were tumor necrosis factor, secreted proteinases, and oxygen metabolytes.

Tumor necrosis factor (TNF) is produced by macrophages after stimulation by LPS and or IFN (11-13). TNF has activity against a range of tumor cells and parasites. The production of TNF mRNA by bone marrow culture derived macrophages after stimulation with various doses of LPS was investigated using a probe which contained a 1700 bp fragment of TNF cloned into the polylinker of PUC 9. The dose response parallels the induction of activation for cytolysis of virally infected cells (Table 8). Further, the production of TNF mRNA returned to background levels after 24 hours of

stimulation with LPS. There is a corresponding lose of activity of macrophages exposed to this dose of LPS for 24 hr for cytolysis of virally infected cells. Thus it appears that TNF may be involved in the cytolysis of virally infected cells. This circumstantial evidence has been confirmed by direct comparison of the sensitivity of infected and uninfected cells to rTNF (Figure 2). Uninfected BALB/c3T3 cells and P815 cells are relatively resistant to lysis by rTNF. BALB/c3T3 cells infected with either VSV or VEE are more sensitive to lysis by rTNF. VEE-infected cells are the most sensitive, parralleling the sensitivity of these two target in the macrophage mediated cytotoxicity assay. Finally, added anti-TNF antibody inhibited macrophage mediated cytolysis of virally infected cells (Table 9).

Table 8. Tumor Necrosis Factor mRNA production in response to LPS Stimulation.

LPS (ng/ml)[1]	TNF mRNA[2]
[illegible]	[illegible]

[1] Bone marrow culture derived macrophages were exposed to the indicated concentrations of LPS for 4 h and then placed on ice to slow metabolism. The RNA was extracted as described in Materials and Methods and dot blotted onto nitrocellulose filter.
[2] The dried filter was probed with a ^{32}P-labelled TNF sequence containing probe. The filter was dried and exposed to X-ray film. The film was developed and the intesities of the dots determined by scanning densitometry. THe results are expressed in relative intensity units with the control value set to 1.

Table 9. Inhibition of Macrophage Lysis of Virally Infected Cells By Anti-TNF.

Stimuli[1]	Percent Specific ^{51}Cr-Release[2]	
	Control	Anti-TNF[3]
LPS (ng/ml)		
10	100	11
1	98	3
0.1	98	0
0.01	17	1
500 IRU α/β-IFN + 10 ng LPS	85	0

[1] Bone marrow culture derived macrophages were exposed to the listed stimuli for 4 h, washed and cocultured with ^{51}Cr-labelled BALB/c3T3 cells that had been infected with VSV. After 16 h incubation specific release was determined.
[2] Specific release = experimental - spontaneous / maximum -spontaneous. Each determination is the mean of triplicate wells.
[3] Rabbit anti-murine TNF-α at 2 x 10^3 neutralizing units per well added with targets.

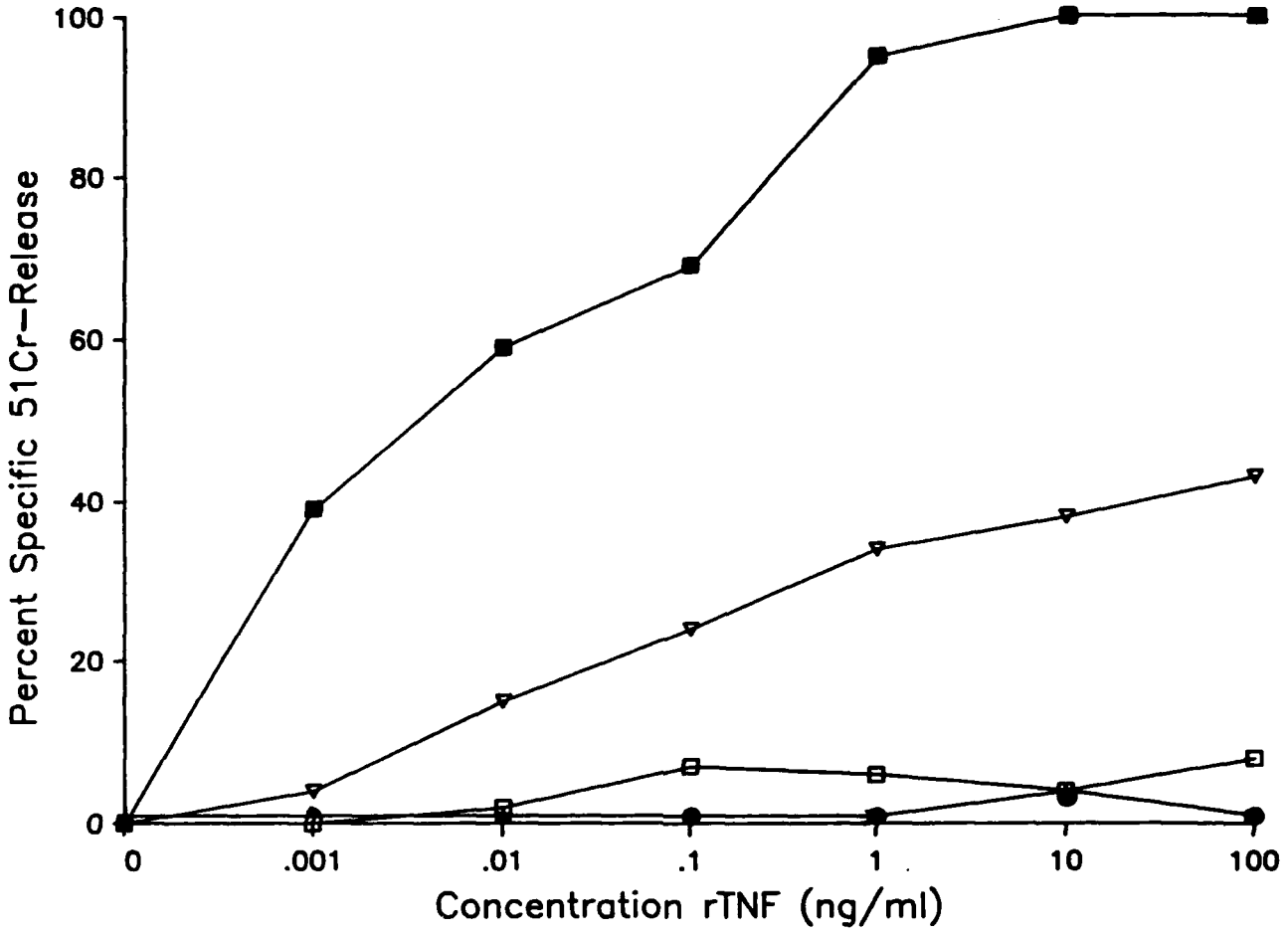


Figure 2. Lysis of Target Cells by rTNF. ^{51}Cr-labelled target cells were incubated with various concentrations of rTNF for 16 h. The specific release was calculated as described. Targets were P815 (●), BALB/c3T3 (□), BALB/c3T3 infected with VSV (■), and BALB/c3T3 infected with VEE (△).

A second mechanism of cytolysis by macrophages for tumor cells is the production of novel proteinases (14-16). We investigated the relative role of proteinases in the observed cytolysis of tumor cells and virally infected cells through the use of various proteinase inhibitors. N-p-tosyl-L-lysine chloromethyl ketone (TLCK) was able to inhibit the lysis of P815 target cells to a much greater extent than VSV-infected BALB/c3T3 cells (Fig. 3). Phenylmethyl sulfonyl fluoride showed slight inhibition of P815 cytotoxicity but no inhibition of VSV-infected BALB/c3T3 cytotoxicity. Bovine pancreatic trypsin inhibitor had no effect on macrophage cytotoxicity against either targets. Thus it appears that proteinases play a much smaller role in the lysis of virally infected target cells than in the lysis of tumor target cells.

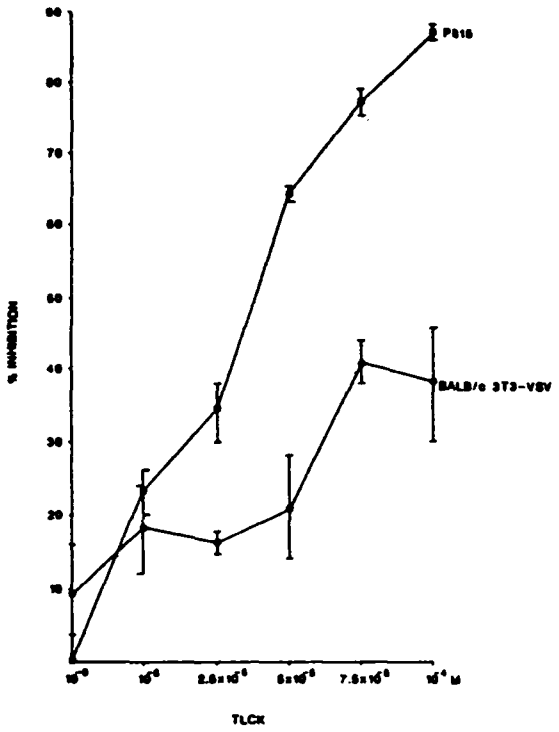


Figure 3. Effect of TLCK on Macrophage Cytotoxicity. Monolayers of bone marrow culture-derived macrophages were activate by a 4 hr exposure to a mixture of 10 IRU/ml α/β-IFN. 1 IRU/ml τ-IFN. and 10 ng/ml LPS. The activated macrophages were then tested for their ability to lyse P815 of VSV-infected BALB/c3T3 cells in the presence of increasing concentrations of TLCK. Lysis was measured in a 16 hr chromium release assay and the data expressed as percent inhibition of cytotoxicity of the respective targets in the absence of TLCK.

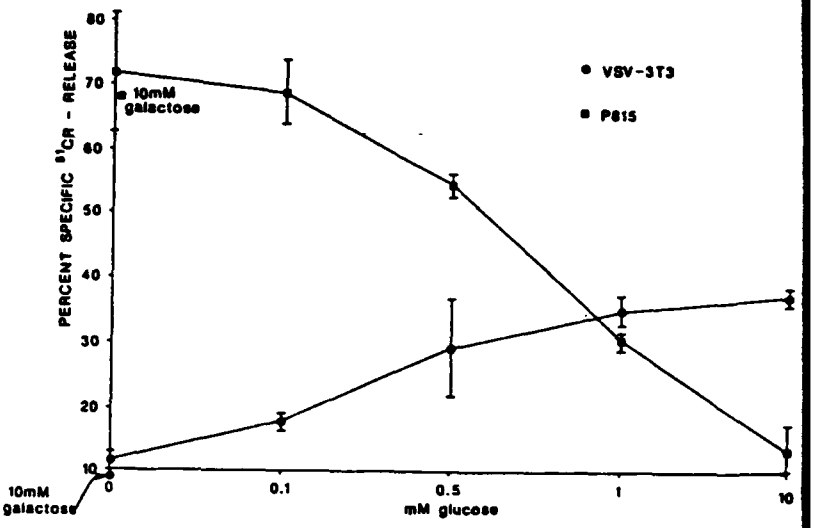


Figure 4. Effect of Glucose Depletion on Macrophage Cytotoxicity. Monolayers of bone marrow culture derived-macrophages were activated by exposure to a mixture containing 10 IRU/ml α/β-IFN. 1 IRU/ml τ-IFN. and 10 ng/ml LPS. The activated macrophages were then tested with 16 hr chromium release assay for their ability to lyse P815 or VSV-infected BALB/c3T3 cells in glucose depleted medium supplemented with increasing concentrations of glucose or galactose. Data is expressed as percent specific release.

Oxygen metabolites

The macrophage generates a series of toxic oxygen metabolites through hexose monophosphate shunt. In an attempt to define the role of these metabolites in the observed cytotoxicity, we ran our assays in hexose-depleted medium. The results are shown in Fig 4. Inhibition of the hexose monophosphate shunt by depletion of glucose from the assay medium was able to significantly decrease the cytotoxicity of macrophages for VSV-infected BALB/c3T3 cells but not P815 target cells. In fact, P815 targets were more susceptible to lysis under glucose depleted conditions. The addition of 10 mM galactose instead of glucose was not able to either restore macrophage cytotoxicity for virally infected targets or to protect P815 targets. Again the mechanism of cytotoxicity for virally infected targets appears to be different from that seen with tumor target cells.

Bermudez and Young (17) have reported that TNF induces increased production of superoxide anion by macrophages and that this is further potentiated by treatment of the macrophages by either IFN or IL-2. This would correlate with the results above and might indicate that the role of TNF may be either directly cytotoxic for the virally infected target cell or may be an autocrine factor which induces production of toxic oxygen metabolite, which in turn are toxic for the virally infected target cell.

Measurement of Virus Specific Cytotoxic T lymphocyte Activity.

From the above work, it is obvious that measuring macrophage activation for tumor cell killing is not the same as measuring macrophage activation for killing virally infected cells. Therefore, we attempted to generate a system to measure the generation of virus (VEE) specific CTL activity. The initial step was to select a time after viral infection to measure the response. To simplify interpretation, we wish to select a time point where NK activity had returned to near normal levels. Mice were injected subcutaneously with TC-83 strain of VEE at different times before sacrifice. The mice were sacrificed on the same day and their spleen cells tested for the ability to mediate virus specific kill. The results are shown in Figure 5. Panel A shows cytotoxicity against cells infected with the homologous virus. Panel B shows cytotoxicity against cells infected with a different virus. Day 9 had the highest level of kill in both panels. There may be greater than normal levels of NK activity remaining at Day 9. Therefore, we decided to use day 11 for further tests.

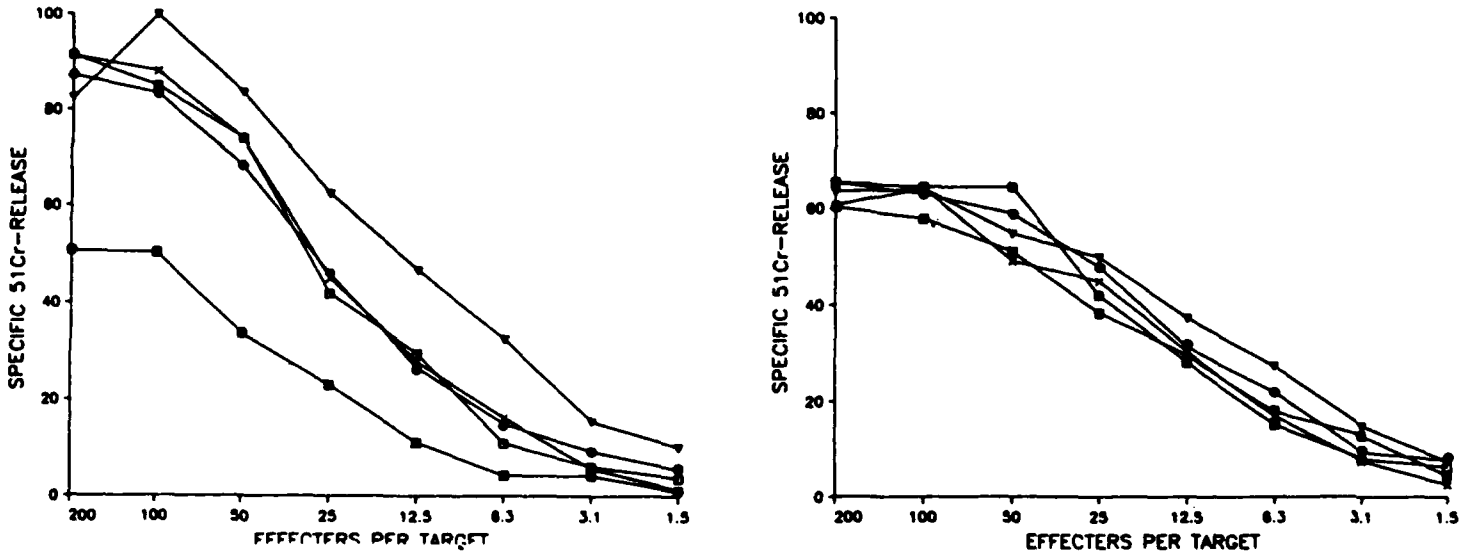


Figure 5. Time Course of Virus Specific CTL Activity. Groups of C3H/HeN mice were injected with 10^4 pfu of TC-83 strain VEE, sc, 9 (▽), 11 (X), 13 (□), and 15 (●) days before sacrifice. One group was left unchallenged (■). Mice were sacrificed and their spleen cells isolated and tested in a 16 h ^{51}Cr-release assay against L-929 cells infected with VEE (Panel A) or infected with VSV (Panel B).

In addition to anti-viral CTL responses, we measured the effect of drug administration on an allogeneic CTL response. Thus groups of C3H/HeN mice were given either drug or a placebo. Mice from each group were then challenge with TC-83 strain of VEE, P815 (cell line allogeneic to mice), or left unchallenged. Spleens were harvested from each group, pooled, counted and reacted with various target cells. Table 10 shows the average spleen cell yield for each treatment group. All groups of mice for an experiment for a given drug were of the same age and sex. There was some variation in age of mice used between experiments.

Table 10. Average Spleen Cell Yield from Drug Treated Mice[a].

Treatment Challenge[b]	Placebo	Drug	Placebo	Drug	Placebo	Drug
	Nothing		P815		VEE	
AVS#	Cells / spleen X 10^{-7}					
1761	3.9	5.9	7.7	7.7	4.4	5.9
1968	3.7	6.7	5.2	10.2	6.4	8.9
2149	4.2	5.3	5.8	4.8	1.7	1.3
2776	3.6	4.5	9.2	5.7	5.9	5.0
2777	3.1	4.7	6.5	5.1	4.4	6.2
2778	4.8	4.3	6.3	6.5	4.4	5.7
3960	4.0	4.7	7.2	8.6	5.5	4.5
4286	4.1	6.0	7.2	6.7	4.6	6.2
4287	3.6	4.3	4.1	4.5	3.5	3.8
4553	5.3	7.1	8.0	10.9	7.4	7.5

[a] Groups of 5 C3H/HeN mice were treated with drug or placebo and then challenged with antigen. Eleven days later mice were sacrificed. The spleens removed and pooled by group. A single cell suspension was prepared from each pool and counted.
[b] Mice were challenged with nothing; 1 X 10^{6} P815 cell, ip; or 10^{4} pfu TC-83 strain of VEE, sc.

In general treatment with a drug did not cause a significant increase or decrease in the number of spleen cells recovered as compared to placebo treated mice challenged in a similar manner. One major exception was AVS 1968. Mice treated with AVS 1968 yielded more cells than plecebo treated mice challenged with the same antigen. Other drugs caused some change in the number of cells recovered but not to the extent of AVS 1968. Thus even with similar levels of cytotoxicity at the per cell level from drug and placebo treated mice, there were more effectors available in the AVS 1968 treated spleen.

Figures 6A-D shows the cytotoxic activity of spleen cells from AVS 2149 or palcebo treated mice against various target cells. Panel A shows the cytotoxic response against the virus specific, histocompatible target. There is clearly an increase in the cytoxic activity of spleen cells isolated from TC-83 infected mice as compared to uninfected controls. When you compare the activities of these same cell populations against the same cells infected with a different virus (VSV), there is no increase in activity due to VEE challenge. The anti-VEE response is increased in drug treated, VEE challenged mice as compared to placebo treated VEE challenged

mice. There was a slight increase seen using virally nonspecific targets. Figures 6C and D look at the antiallogeneic response. There is a decrease in activity placebo treated to drug treated groups. Thus for this drug, treatment did not result in a change in the total number of cells recovered from the spleens but an increase in the per cell anti-virus CTL but a decrease in the anti-allogeneic responses. However, AVS 2149 has not had any effect on the survival of mice challenged with VEE.

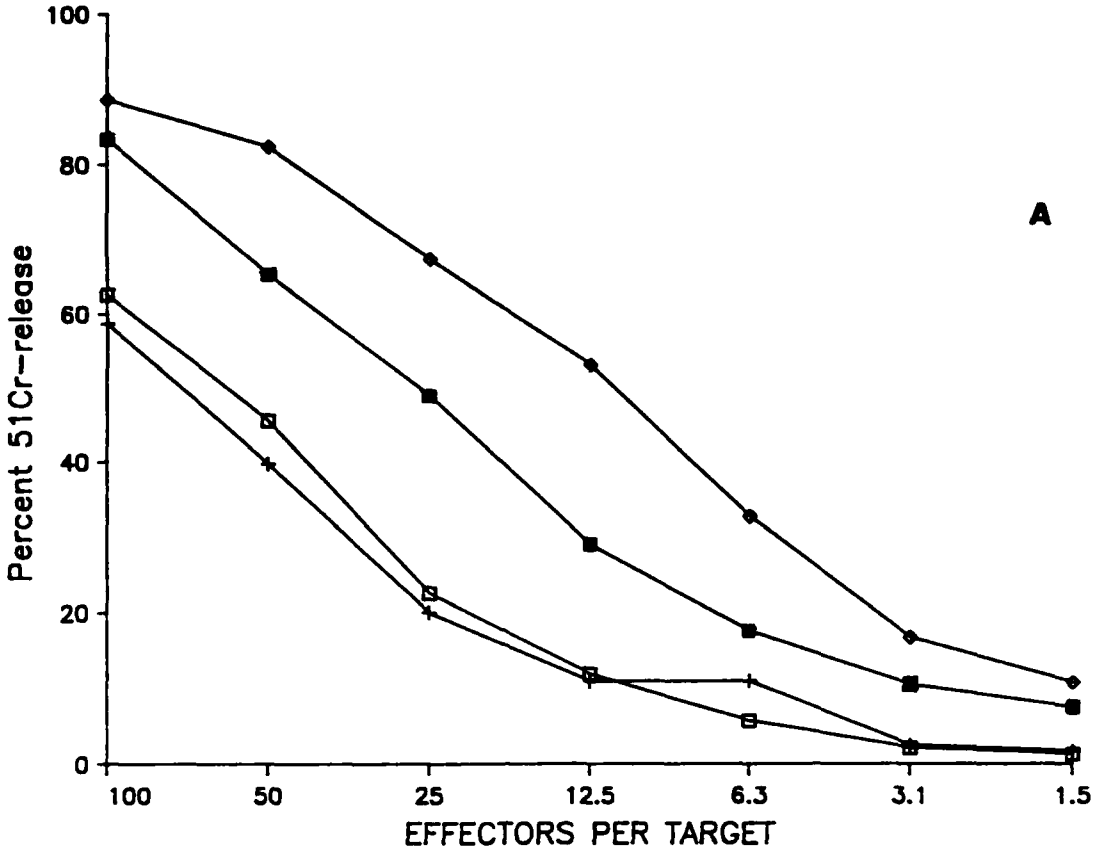
Similar data has been accumulated for 9 other drugs. For simplicity the data has been summarized in Table 11. This table does not attempt to signify the magnitude of the change. A change is simply decernable at all or most all of the E:T ratios tested.

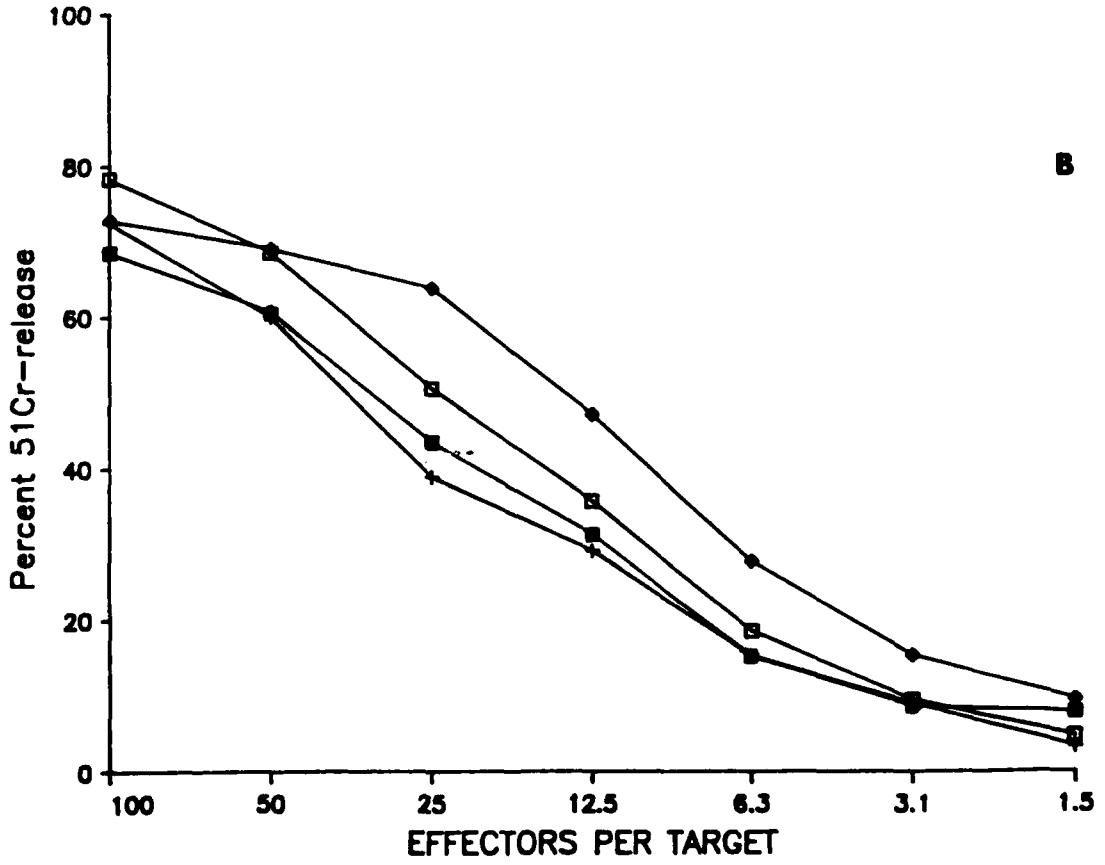
Table 11. Summary of the Effect of Drug Treatment on Anti-virus and Anti-allogeneic CTL Repsonses.

Drug	Virus	Allogeneic
1761	Decrease	No Change
1968	Decrease	No Change
2149	Increase	Decrease
2776	No Change	No Change
2777	No Change	No Change
2778	No Change	Increase
3960	No Change	No Change
4286	Decrease	Decrease
4287	No Change	Decrease
4593	No Change	Decrease

The take home message of this table is that measuring the anti-virus and anti-allogeneic responses do not always result in the same answer. Also the ability to modulate either response does not appear to positively correlate with the in vivo protective activity of the drug for protections against lthal challenge with VEE. In fact, the two drugs that have in the past shown protection against VEE appear to decrease the specific anti-VEE CTL resposes.

Figure 6. Effect of AVS 2149 Treatment on Development of Antiviral and Antiallogeneic CTL Activity. Groups of 5 mice each were treated by intraperitoneal injection of either AVS-2149 (4 mg/kg) or PBS on day -1 with regard to antigen challenge. Mice were challenged by the subcutaneous injection of 10^5 pfu TC-83 passed once in chicken embryo cell culture in the presence of 4% chicken serum or the intraperitoneal injection of 1×10^6 P815 cells washed in serum-free medium to remove serum antigens. Control mice were left uninjected. Eleven days after antigen challenge, mice were sacrificed and each group used to prepare a pool of spleen cells: PBS alone (+), drug alone (□), PBS plus P815 (●), drug plus P815 (▽), PBS plus TC-83 (■), drug plus TC-83 (◇). These were tested at various concentrations against a constant number of ^{51}Cr-labelled target cells. The target cells were A) L929 infected with TC-83 at an moi of 3, B) L929 infected with VSV at an moi of 3, C) BALB/c3T3 infected with TC-83 at an moi of 3, and D) P815. Specific release was determined by the formula: Specific release = (experimental release - spontaneous release) / (maximum release - spontaneous release). Each value represents the mean of triplicate determinations.


A
Percent 51Cr-release
100
80
60
40
20
0
100
50
25
12.5
6.3
3.1
1.5
EFFECTORS PER TARGET


B
Percent 51Cr-release
100
80
60
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25
12.5
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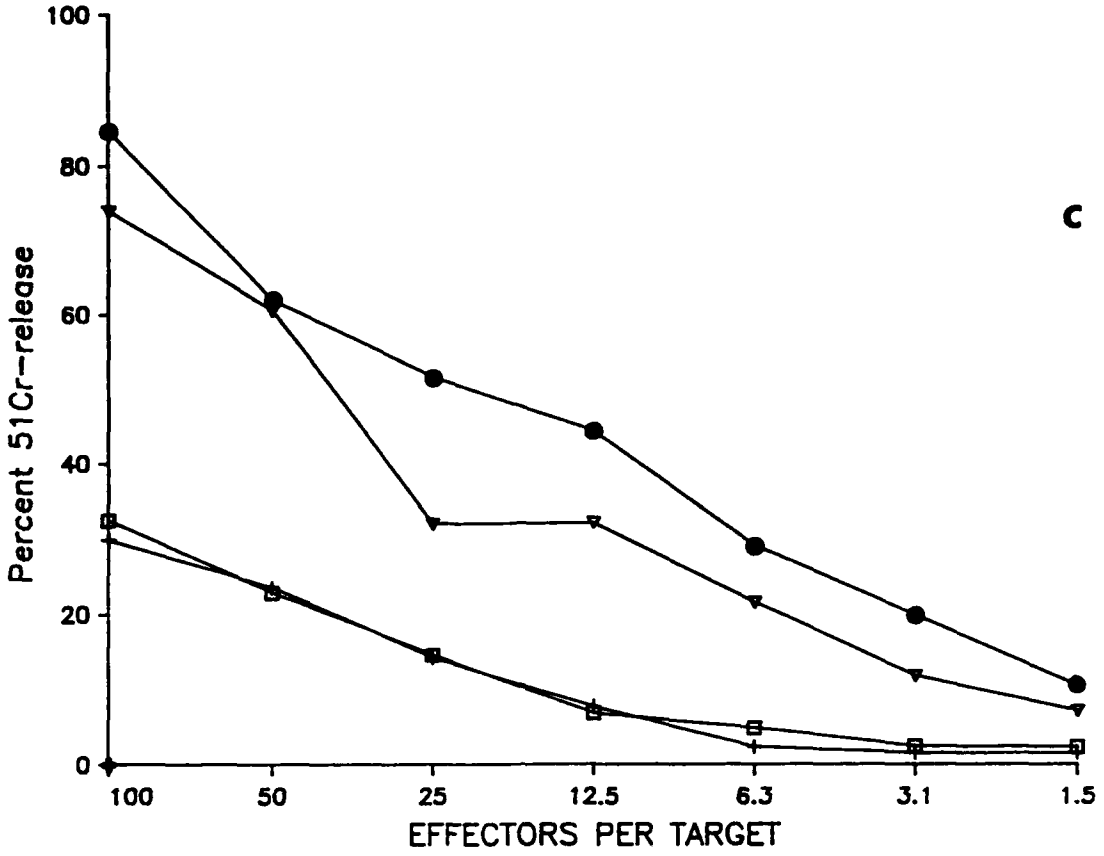
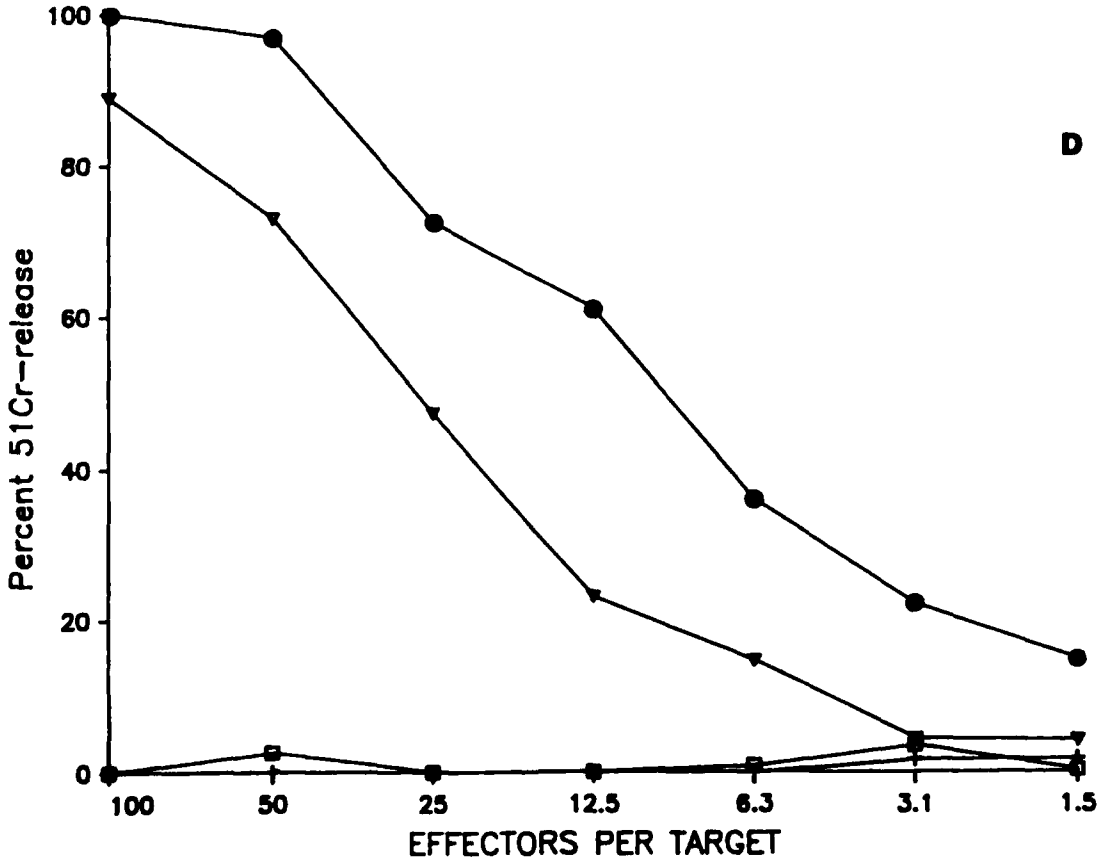
EFFECTORS PER TARGET


C
Percent 51Cr-release
100
80
60
40
20
0
100
50
25
12.5
6.3
3.1
1.5
EFFECTORS PER TARGET


D
Percent 51Cr-release
100
80
60
40
20
0
100
50
25
12.5
6.3
3.1
1.5
EFFECTORS PER TARGET

In vitro Testing of the Combination of AVS 1968 and F68 for Anti-VEE Activity.

AVS 1968 (CL246,738) has shown effectivenss in prolonging the lives of mice infected with the 68U201 strain of VEE. However, the end result is still death (18/20). Compound F68 was obtained from CytRx Corporation. This is a block polymer preparation that is in IND trials for treatment of escemia. We reasoned that the F68 might have an effect on the blood-brain barrier, allowing CL246,738 to be more active in the brain. If were able to increase to the immune acivity in the brain it was thought that the the animals might survive the infection. The results of an initial experiment are shown in Table 11.

Table 12. Antiviral activity of a mixture of CL246,738 and F68 against Venezuelan equine encephalitis virus strain 68U201.

	Cumulative Deaths[a]										
Days post virus	0	8	9	10	11	12	13	31	Dead/injected	MTD	MST
Treatment[b]											
F68 + CL246, 738 ip	10										
F68 + CL246, 738 orally	1	2	2	3	3	5	5	5	5/9	13.4	12
saline		2	4	7	10				10/10	9.7	10

[a] Mice were observed daily. The experiment was terminated on day 31. Surviving mice were healthy at time of sacrifice.
[b] CL246, 738 and F68 were prepared in pyrogen free saline at 40 or 4 mg/ml respectively. Equal volume of each solution was then mixed. The mixture (0.5 ml) was administered by either intraperitoneal injection or orally through a feeding tube. Twenty-four hours later the mice were challenged with a subcutaneous injection of 400 pfu of 68U201 strain VEE.

The ip administration of the mixture of the drug proved to be toxic. At the doses used, these drugs by themselves have not shown any toxicity. With oral administration the combination gave the best survival percentage of any treatment yet attempted. This might be improved by lowering the drug toxicity. However, due to the shortage of the drug and the fact that there was not complete protection, we have discontinued this work. We would be willing to look into optimizing the treatmnet regimen if the technical representitive feels that it is advantageous and if more drug is available.

Selection of Plaque type Variants of Pichinde Virus.

We have previously reported (18) the isolation of plaque type variants of Pichinde strain An Co 3739 isolated from progeny of cocultures of Vero and L-929 cells. Similar plaque type variants have also been isolated from a second strain of Pichinde virus (Co 4763). As with the previous isolates these isolates maintain plaque morphology through repeated passage in mouse

L-929 cells. The differences in plaque morphology were not only in size but in the extent of cytopathology observed. These variants are presently being tested for lethality in mice. Even if one does not prove to be a mouse lthal Pichinde suitable as a model for Lasa fever, they offer a system for the study of the cellular events involved in pahtology of this virus.

LITERATURE CITED

1. Levine, J., Tomasulo, P.A., and Oser, R.S. Detection of endotoxin in human blood and demonstration of an inhibitor. J. Lab. Clin. Med. 75:910, 1971.
2. Scheele, W.P. Inapparent viral infection of cells in vitro. I. Conversion of inapparent to apparent infection by environmental alteration of chicken embryonic cells in cultures inoculated with Japanese encephalitis virus. Amer. J. Path. 47:181, 1964.
3. Leong, T.C., Russell, P.W., LeBlanc, P.A., and Challberg, S. [illegible] murine macrophages cultured from mouse bone marrow. Cell Tiss. Res. 230:611, 1983.
4. Johnson, K.M., Sherman, R.W., and Hanford, [illegible] geographical distribution of Venezuelan encephalitis virus in Guatemala, Honduras and British Honduras during 1965-68, and its possible movement to Central America and Mexico. Am. J. Trop. Med Hyg. 18:703, 1969.
5. Trapido, H. and Sanmartin, J. Pichinde virus: a new virus of the Tacaribe group from Columbia. Am. J. Trop. Med. Hyg. 20:631, 1971.
6. Jahrling, P.B., Hesse, R.A., Rhoderick, J.B., Elwell, M.A., and Moe, J.B. Pathogenesis of a Pichinde virus strain adapted to produce lethal infections in guinea pigs. Infect. Immun. 32:872, 1981.
7. Russell, S.W. Quantification of cytolysis of neoplastic cells by release of chromium-51. In Methods for Studying Mononuclear Phagocytes. (Adams, D.O., Edelson, P.J., and Koren, H., Eds.) New York: Academic Press, p. 799, 1981.
8. Pace, J.L., Russell, S.W., LeBlanc, P.A., and Murasko, D.M. Comparative effects of various classes of mouse interferons on macrophage activation for tumor cell killing. J. Immunol. 134:977, 1985.
9. Blalock, J.E., and Gifford, G.E. Inhibition of interferon action by vitamin A. J. Gen. Virol. 29:313, 1975.
10. Maniatis, T., Fritsch, E.F., and Sambrook, J. Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.
11. Old, L. Tumor necrosis factor (TNF). Science 230:630, 1985.
12. Chen, A.H., McKinnon, K.P., and Koren, H.S. Lipopolysaccharide (LPS) stimulates fresh human monocytes to lyse actinomycin-D-treated WEHI-164 target cells via increased secretion of a monokine similar to tumor necrosis factor. J. Immunol. 135:3978, 1985.
13. Philip, R. and Epstein, L.B. Tumor necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, γ-interferon, and interleukin-1. Nature 323:86, 1986.
14. Adams, D.O. Effector mechanisms of cytolytically activated macrophages. I. Secretion of neutral proteinases and effect of proteinase inhibitors. J. Immunol. 124:286, 1980.

15. Adams, D.O., Kao, K.J., Farb, R., and Pizzo, S.V. Effector mechanisms of cytolytically activated macrophages. II. Secretion of a cytolytic factor by activated macrophages and its relationship to secreted neutral proteinases. J. Immunol. 124:293, 1980.
16. Hibbs, J.B.,Jr., Taintor, R.R., Chapman, H.A.,Jr., and Weinberg, J.B. Macrophage tumor killing: influence of the local environment. Science 197:279, 1977.
17. Bermudez, L.E.M. and Young, L.S. Tumor necrosis factor, alone or in combination with IL-2, but not IFN-γ, is associated with macrophage killing of Mycobacterium avium complex. J. Immunol. 140:3006, 1988
18. LeBlanc, P.A. and Winters, A.L. (1988) U. S. Army Medical Research and Development Command Annual Report on Contract No. DAMD17-86-7-6121.

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